Report for 2005AK45B: Characterizing sources and growth potential of indicator bacteria in cold region streams

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Report Follows

Antibiotic Resistance Analysis of *Enterococci* in Chester Creek

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Abstract

Antibiotic Resistance Analysis (ARA) is a technique that can be employed to identify the source of fecal indicator bacteria in rural and urban watersheds. In this ongoing study, ARA is being utilized to investigate the sources of *Enterococcus* bacteria in Chester Creek, Anchorage, AK. Possible sources of fecal bacteria in the Chester Creek Watershed include waterfowl, moose, bear, beaver, domestic animals, and sewer/septic inputs. Thus far, 170 isolates have been collected and used for ARA. Results to date indicate that the antibiotic resistance of unknown isolates increases with downstream distance. Isolates originating from moose have shown to be resistant to only five of the eleven antibiotics tested, and indicate that antibiotic resistance in moose may depend on the age of the animal. Canine isolates have shown to be primarily resistant to four of the eleven antibiotics tested including CEP, GEN, KAN, and STR. Further isolate testing using ARA is ongoing and more complete results will be presented at the conference.

Introduction

Fecal contamination is a problem currently being faced in many urban and rural watersheds. Fecal pollution can lead to disease outbreaks and regulatory closure of surface water bodies to recreation and other activities. Efforts to detect fecal indicator organisms are easily achieved, but tracking fecal indicator sources has proved to be much more difficult. Microbial Source Tracking (MST), also known as Bacterial Source Tracking (BST), refers to a group of analytical techniques that can be used to trace the origins of fecal indicator bacteria such as *Eschericia coli* (Scott et al., 2002; Simpson et al., 2002). Antibiotic Resistance Analysis (ARA) is a phenotypic MST technique that can provide reliable results regarding the origination of microbial pollutants. It has been studied and applied in numerous locations such as Virginia, Florida, and California (Graves et al., 2002; Jiang, 2003). Presently, ARA has not been investigated for use in extremely cold climates such as Alaska. The ARA technique is based upon the premise that fecal bacteria in humans and animals differ in their antibiotic sensitivity due to different levels of exposure encountered throughout an animal's life. This difference allows for a library style classification scheme using multi-variate statistical techniques such as discriminant analysis.

Background

Anchorage area streams experience a considerable fecal load from wildlife, domestic animals, and human sources. Twelve of the Municipality of Anchorage's bodies of water are contaminated with fecal coliforms (ADEC, 2003). In addition, Chester Creek is listed on the EPA Clean Water Act under Section 303(d) for contamination by fecal coliforms (Rice et al., 2003). The creek has been studied for years without yielding a decrease in fecal coliform levels, and efforts to study and characterize the fecal coliform problem are ongoing through numerous local, state, and federal agencies.

Potential sources of fecal pollution in Anchorage area streams include waterfowl, moose, bear, beaver, domestic animals, and sewer/septic inputs. Efforts to track the source of this pollution using either phenotypic or genotypic methods of MST have not previously been undertaken.

This study seeks to employ ARA to identify the primary contributors to the fecal coliform load in Chester Creek. This information will allow more informed decisions to be made regarding Best Management Practices (BMPs) for Chester Creek and other Anchorage watersheds contaminated with fecal coliforms.

Project goals are being pursued through the collection and analysis of water samples from five locations in Chester Creek. The first sampling location on Fort Richardson Army Base is relatively pristine with no development upstream of this site. The second and third sites are located at the inlet and outlet to University Lake, a popular recreation area located in a more urbanized area of the stream. University Lake also serves as a no-leash dog park, and fecal contributions from canines are being thoroughly explored at these two locations. There is also considerable channelization between the Fort Richardson site and University Lake, which could impact fecal coliform concentration and survival. The fourth sampling location is at the University of Alaska Anchorage, downstream of two hospitals as well as University Lake. The final sampling location is near Arctic Boulevard, where channelization and development are extensive.

Fecal coliforms as a group are not amenable to ARA due to the wide variety of bacteria that are encompassed within the classification. Instead, ARA is being performed on a fecal coliform subgroup from the genus *Enterococcus* due to their ease of acquisition, culture, and the availability of supporting literature (Wiggins et al., 2003).

Materials and Methods

Samples for Enterococci enumeration are obtained at least bimonthly and processed for use in ARA. A complete schematic of the ARA procedure can be seen in Figure 1. Enterococci samples are collected in sterile 125 ml whirl-pak bags, and immediately transported to the laboratory on gel ice for analysis. Samples are filtered in 20, 50, and 100 mL aliquots through sterile filter funnels (Pall MicroFunnel), with 0.45-µm Gelman GN-6 filters. Filters are then transferred to 50 mm Petri dishes containing mEnteroccocus agar (Difco) and incubated at 37°C for 48 hours. Individual isolates of *Enterococci* appear as red dots and are then transferred using sterile toothpicks into sterile 96-well micro-titer plates (NUNC) filled with 0.2 ml of Enterococcosel broth (BBL). The 96-well plates are incubated for an additional 48 hours at 37°C. A dark brown color in the well indicates a positive response to the esculin catalase test, and these samples are employed for ARA analysis. Isolates that do not hydrolyze esculin (i.e., produce a dark brown color) are not considered to be enterococci and are discarded.

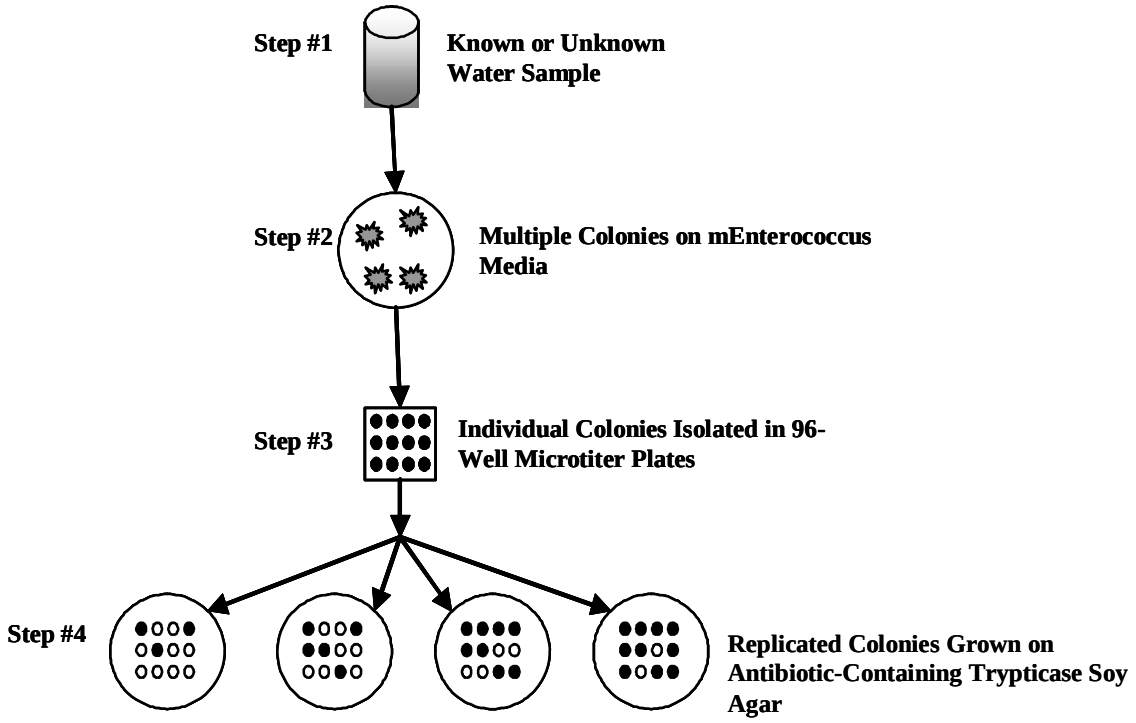

Figure 1. Schematic of ARA procedure.

Based on previous work by Wiggins, eleven antibiotics are used to test the isolates. These include bacitracin (BAC, Sigma), cephalothin (CEP, Sigma), chlortetracycline hydrochloride (CTC, Sigma), erythromycin (ERY, Sigma), gentamicin (GEN, Sigma), kanamycin monosulfate (KAN, Sigma), neomycin sulfate (NEO, Sigma), oxytetracycline hydrochloride (OTC, Sigma), streptomycin sulfate (STR, Sigma), tetracycline (TET, Sigma), and vancomycin (VAN, Sigma). Antibiotic plates are prepared in Trypticase Soy Agar (BBL) in the following concentrations: 10, 25, 50, and 100 µg/ml BAC; 10, 15, and 50 µg/ml CEP; 20, 60, and 80 µg/ml CTC; 10, 30, and 50 µg/ml ERY and TET; 5, 10, and 20 µg/ml GEN; 10, 15, 30, and 50 KAN and NEO; 20, 40, and 80 µg/ml OTC; 20, 40, 60, and 80 µg/ml STR; 5, 10, and 30 µg/ml VAN (Wiggins, 2003).

Following a positive response to the esculin catalase test, bacterial isolates are transferred to 100 mm sterile Petri dishes containing the various antibiotic concentrations in TSA using a 48-prong replica-plater (Sigma). For each test, there are a total of 37 plates containing TSA with antibiotics and two blank plates containing TSA with no antibiotics. To test for the possibility of antibiotic cross contamination, one blank is replica-plated before and after the replica-plating of the antibiotics.

Resistance to antibiotics is determined by comparison with the isolates grown on the plates containing no antibiotics. Isolates that show decreased growth are considered to be sensitive to that concentration of antibiotic. A spreadsheet showing various isolates and their resistances can be seen in Table 1.

Library generation is performed by collecting fresh fecal samples from within the Chester Creek Watershed boundaries as defined by the USGS and the Municipality of Anchorage (Rice et al., 2003). The fecal material is mixed with a sterile saline buffer in amounts varying

from 0.1 – 1.0 g. The samples are filtered as previously described for water samples in aliquots of 20, 50, and 100 ml. When possible, fecal samples are collected within two hours of deposition. Human samples will be collected from the Municipality's water treatment plant as well as from septic pump trucks. ARA is performed as specified above.

Discriminant analysis will be performed using the SAS statistical software. As different combinations of antibiotics are expected to result in different Average Rate of Correct Classification (ARCC), multiple combinations of antibiotics will be analyzed to determine the most appropriate discriminant variable (Wiggins, 1996).

Classification using discriminant analysis will be performed with respect to three different classification schemes. The first is the human vs. non-human classification scheme. This is expected to quantify the relative input from any septic or sewer sources. The second classification scheme, termed "management level classification scheme," sorts isolates into human, domestic animals, or wildlife categories. This is the most useful classification scheme of the three as it is anticipated to provide enough information to allow better BMPs to be established. The majority of the statistical analysis performed will be aimed at establishing a good management level classification with a high ARCC. The third classification scheme is a species level classification and can provide information into what particular species are polluting the creek (i.e. differentiation between ducks, geese, moose, dogs, etc.). As ARCCs for species level classification have historically been low using ARA (50-70%), results are not anticipated to provide conclusive species level discrimination (Wiggins et al., 1999).

In addition to the three different classification schemes, discriminant analysis will be performed for specific sites, at different times of the month or year, and at all locations and all sampling times. This will allow for the determination of source changes related to time or downstream distance.

Results and Discussion

This study is currently ongoing. Thus far, 170 isolates have been cultured under the antibiotic regimen described above. A total of 1100 additional isolates are planned for development of the library and determination of the discriminant variables.

Preliminary results from unknown samples have shown that in general, antibiotic resistance of *Enterococci* bacterial isolates increases with downstream distance, with the highest antibiotic resistances observed near the University Lake and University of Alaska Anchorage, as well as downstream at the Arctic site.

Isolates analyzed from a female adult moose were observed to have some resistance to KAN, NEO, and BAC, but very little resistance to other antibiotics.
Isolates obtained from a calf, however, displayed little resistance to antibiotics other than KAN, NEO, and STR. It is possible then, that the age of the animal influences the ARA profile of the indicator bacteria, due to different exposures throughout the animal's life.
Isolates analyzed from canine fecal samples have been observed to have some antibiotic resistance to CEP, GEN, KAN, and STR but little resistance to other antibiotics. Table 1 shows six isolates from three known sources and their resistances to the various antibiotics.

	Maximum antibiotic concentration showing growth (µg/ml)											
Source	BAC	CEP	CTC	ERY	GEN	KAN	NEO	OTC	STR	TET	VAN	BLANK
Female Moose	10	NG	NG	NG	NG	10	15	NG	NG	NG	NG	G
Female Moose	NG	NG	NG	NG	NG	10	15	NG	NG	NG	NG	G
Female Moose	10	NG	NG	NG	NG	50	15	NG	20	NG	NG	G
Female Moose	10	NG	NG	NG	NG	30	15	NG	NG	NG	NG	G
Female Moose	10	NG	NG	NG	NG	10	15	NG	NG	NG	5	G
Female Moose	10	NG	NG	NG	NG	30	15	NG	NG	NG	NG	G
Calf (Moose)	NG	NG	NG	NG	NG	30	NG	NG	NG	NG	NG	G
Calf (Moose)	NG	NG	NG	NG	NG	30	NG	NG	NG	NG	NG	G
Calf (Moose)	NG	NG	NG	NG	NG	50	15	NG	20	NG	NG	G
Calf (Moose)	NG	NG	NG	NG	NG	50	15	NG	20	NG	NG	G
Calf (Moose)	NG	NG	NG	NG	NG	50	15	NG	20	NG	NG	G
Calf (Moose)	NG	NG	NG	NG	NG	30	15	NG	NG	NG	NG	G
Dog	NG	NG	NG	NG	NG	30	NG	NG	20	NG	NG	G
Dog	NG	15	NG	NG	5	50	NG	NG	20	NG	NG	G
Dog	NG	15	NG	NG	5	50	NG	NG	20	NG	NG	G
Dog	NG	15	NG	NG	5	50	NG	NG	20	NG	NG	G
Dog	NG	15	NG	NG	5	50	NG	NG	20	NG	NG	G
Dog	NG	15	NG	NG	5	50	NG	NG	20	NG	NG	G

NG- No Growth G- Growth

Table 1. Antibiotic resistance of various *Enterococcus* isolates.

These general antibiotic resistance profiles will be used in conjunction with many profiles yet to be obtained to identify which antibiotics will be used as discriminant variables in the discriminant analysis. This will allow more informed decisions to be made regarding the management of Chester Creek and other Anchorage bodies of water contaminated with fecal coliforms.

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